

Europäisches Patentamt

European Patent Office

Office européen des brevets



(11)

EP 0 550 162 B1

(12)

EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention
of the grant of the patent:
15.03.2000 Bulletin 2000/11

(51) Int. Cl.⁷: C12N 15/82, C12N 9/02,
A01H 5/00

(21) Application number: 92311125.6

(22) Date of filing: 07.12.1992

(54) Fatty acid alteration by a Delta-9 desaturase in transgenic plant tissue

Änderung von Fettsäuren durch eine Desaturase in transgenischem Pflanzengewebe

Altération d'acides gras par une desaturase dans des tissus transgéniques de plantes

(84) Designated Contracting States:
AT BE CH DE DK ES FR GB GR IE IT LI LU NL PT
SE

(30) Priority: 31.12.1991 US 816288

(43) Date of publication of application:
07.07.1993 Bulletin 1993/27

(73) Proprietor:
University of Kentucky Research Foundation
Lexington, Kentucky 40506-0058 (US)

(72) Inventors:
• Hildebrand, David F.
Lexington, Kentucky 40503 (US)
• Grayburn, W. Scott
Lexington, Kentucky 40503 (US)

(74) Representative:
Mercer, Christopher Paul et al
Carpmaels & Ransford
43, Bloomsbury Square
London WC1A 2RA (GB)

(56) References cited:
EP-A- 0 442 592 WO-A-91/13972
WO-A-91/18985 US-A- 5 057 419

- JOURNAL OF BIOLOGICAL CHEMISTRY vol. 263, 1988, BALTIMORE, MD US pages 2532 - 2535 P.STRITTMATTER ET AL
- JOURNAL OF BIOLOGICAL CHEMISTRY vol. 265, no. 33, 1990, BALTIMORE, MD US pages 20144 - 20149 STUKEY J.E.; MCDONOUGH V.M.; MARTIN C.E. 'The OLE1 gene of Saccharomyces cerevisiae encodes the Delta9 fatty acid desaturase and can be functionally replaced by the rat stearoyl-CoA desaturase gene'
- PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 88, no. 6, 1991, WASHINGTON US pages 2510 - 2514 SHANKLIN J; SOMERVILLE C 'STEAROYLACYL-CARRIER-PROTEIN DESATURASE FROM HIGHER PLANTS IS STRUCTURALLY UNRELATED TO THE ANIMAL AND FUNGAL HOMOLOGS'
- BIOTECHNOLOGY vol. 10, no. 6, June 1992, NEW YORK US pages 675 - 678 GRAYBURN WS; COLLINS GB; HILDEBRAND DF 'FATTY ACID ALTERATION BY A DELTA-9 DESATURASE IN TRANSGENIC TOBACCO TISSUE'

Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

Description**Technical Field**

- 5 [0001] The present invention relates to the alteration of synthesis of fatty acids and the corresponding compositions of triacylglycerols in plants via use of a fatty acid-Coenzyme A or $\Delta 9$ desaturase gene.

Background of the Invention

- 10 [0002] Plants naturally produce an assortment of fatty acids and synthesize an even wider assortment of lipids, including mono-, di- and triacylglycerols, phospholipids, glycolipids, and others, from the fatty acids they produce. The specific assortment of lipids made by any particular plant is determined by both the genotype of the plant and the plant's response to environmental factors such as heat, cold, drought, etc. However, regardless of the environmental conditions, a plant can never produce a fatty acid or lipid composition for which it does not have the necessary biochemical
- 15 machinery, and such biochemical pathways are ultimately determined by genotype. Traditional methods of genetic modification involve genetic recombination processes which are directed by the plant breeder at the whole plant level. These methods, while well characterized and straightforward to conduct, typically produce incremental improvements in oil content and composition by optimizing the native biochemistry, rather than by creating new biochemical pathways.

- [0003] At the same time, because of their influence on food quality and significance in biological processes, there is
- 20 continuing interest in the alteration of fatty acid desaturation mechanisms in plants. The properties of fats and oils are determined by their fatty acid composition, which in turn affects nutritional quality and oxidative stability. Likewise, the specific structures and compositions of other plant lipids which the plant synthesizes from fatty acids are dependent upon the makeup of the fatty acid pool which is available as precursors to the biosynthesis of those lipids.

- [0004] Recently there has been interest in reducing the content of saturated fatty acids in foods. Medical and nutritional research have led many food and food component producers to want certain compositions in their fat and oil
- 25 based foods and food components. Those desired compositions are frequently high in mono- and polyunsaturated fatty acids and corresponding triacylglycerol stores, or are low in saturated fatty acids and saturated fatty acid-based triacylglycerols. Industrial users of plant-derived fats and oils also have preferences for the specifications of feedstocks used in their industrial processes, and such specifications often call for large percentages of a single fatty acid moiety.
- 30 Often the preferred fatty acid moiety is an unsaturated fatty acid moiety such as palmitoleate, oleate, linoleate, or linolenate. Unfortunately, nature does not cooperate by providing oilseed plants which produce the preferred compositions. Efforts have therefore been initiated to develop oilseed varieties and hybrids which yield vegetable oils with higher monounsaturated fatty acid contents. However, in view of the incremental nature of whole-plant genetic methods, the need and desire continue to exist for compositions and methods which can affect and create biochemical pathways at
- 35 the single-gene level through genetic engineering.

- [0005] Even when traditional plant breeding methods are successful in altering fatty acid composition in the lipids of a plant variety, the native biochemical pathways of the plant still exhibit all of their art-recognized characteristics and limitations. Thus, for example, oilseed crops which have been improved by plant breeding exhibit the usual responses to environmental variations. These responses include a tendency to produce higher percentages of saturated fats under
- 40 warmer growing conditions and higher percentages of unsaturated fats under cooler growing conditions, making the reliable production of oilseeds having a particular fatty acid composition as difficult as predicting the weather. Thus, it would also be highly desirable to have means for compensating for these environmental influences. Little effort has been invested to date toward that objective.

- [0006] Finally, there is a continuing desire to improve and extend the environmental range of crop plants. Some
- 45 oilseed species originated in temperate, subtropical and tropical regions and are poorly adapted to cooler production areas. Even oilseeds which are suitable for cooler climates can benefit from further adaptation, since moving up the planting time in the spring and extending the growing season in the summer and fall can sometimes be exploited for higher crop yields. Plant breeders have focused a great deal of attention on one aspect of the necessary climatic adaptation, maturation rate, but another important aspect of climatic adaptation is chilling tolerance (as distinguished from freezing tolerance.)
- 50

Brief Description of the Drawings**[0007]**

- 55 FIG. 1 is a plasmid map of the plasmid used in the transformation work described in Examples 1-5.
- FIG. 2 is a bar graph depicting the results of the experiments of Examples 1-4, showing differences in 16:1/16:0 and 18:1/18:0 ratios in control and transformed callus tissue.

FIG. 3 is a bar graph depicting the results of the experiments of Examples 1-4, showing a computer-generated image from gels showing the presence of the gene of this invention as amplified by PCR.

FIG. 4 is a bar graph depicting the results of the experiments of Examples 1-4, showing differences in 16:1/16:0 and 18:1/18:0 ratios in control and transformed plant tissue.

FIG. 5 is a bar graph depicting the results of the experiments of Examples 1-4, showing differences in 16:0, 18:2 and 18:3 fatty acid percentages in control and transformed plants.

FIG. 6 is a bar graph depicting the results of the experiments of Examples 1-4, showing differences in 16:1, 16:3, 18:0 and 18:1 percentage levels between control and transformed plant tissue.

FIG. 7 is a bar graph depicting the results of the experiments of Examples 1-4, showing differences in 16:1/16:0 and 18:1/18:0 ratios in control and transformed plant tissue.

FIG. 8 is a bar graph depicting the results of the experiments of Examples 1-5, showing alterations of saturated to unsaturated fatty acid ratios in mature seed of the plants transformed according to this invention.

Disclosure of the Invention

[0008] The present invention provides a chimeric expression cassette comprising a cDNA clone which encodes a mammalian fatty acid-Coenzyme A desaturase, operably linked to regulatory sequences which cause the expression of the cDNA clone in plant cells.

[0009] Preferably, the fatty acid-Coenzyme A desaturase gene is a stearyl-Coenzyme A desaturase gene. The stearyl-Coenzyme A desaturase gene is preferably from the rat.

[0010] The expression cassette is optionally capable of being replicated in a bacterial cell.

[0011] The present invention further provides bacterial cells containing as a foreign plasmid at least one copy of an expression cassette as described above.

[0012] There is also provided by the present invention transformed plant cells containing at least one copy of the expression cassette described above. Such transformed plant cells may be of a monocotyledonous species, preferably maize, sorghum, wheat, palm or rice cells. Alternatively, the transformed plant cells may be of a dicotyledonous species, preferably soybean, rapeseed, jojoba, Chinese tallow tree, tobacco, safflower, peanut or sunflower cells.

[0013] The present invention also provides a cell culture or tissue culture comprising cells as described above.

[0014] According to a further aspect of the present invention, there is provided a method of increasing the percentage compositions of unsaturated fatty acid moieties and corresponding fatty acid-derived lipids in plants cells and whole plants, comprising the step of inserting into such plant cells or the cells of such whole plants a chimeric expression cassette as described above. The method may also comprise the further step of sexually or clonally reproducing the whole plants in such a manner that at least one copy of the sequence provided by the expression cassette is present in the cells of progeny of the reproduction.

[0015] Preferably, the expression cassette is introduced into the cells by electroporation, microparticle bombardment or microinjection.

[0016] According to the present invention there is also provided a method for increasing the percentage compositions of unsaturated fatty acid moieties and corresponding fatty acid-derived lipids in *Agrobacterium*-susceptible dicotyledonous plants in which the expression cassette is introduced into the cells by infecting the cells with *Agrobacteria*, a plasmid of which has been modified to include the expression cassette as described above.

[0017] Unsaturated fatty acids are produced by different enzymes in plant and animal cells, and there is little structural or sequence homology between the desaturases found in animal and yeast cells and the corresponding enzymes in plants. However, it has now been found that when a construct according to this invention, containing a gene for a Coenzyme A-dependent desaturase, is introduced into plant cells by a conventional transformation method, such as microparticle bombardment, *Agrobacterium* infection, or microinjection, the gene is expressed in the cells under control of the juxtaposed plant regulatory sequences and successfully interacts with the biosynthetic machinery which is naturally present in the plant cells to catalyze desaturation of the naturally produced palmitate and stearate moieties, yielding palmitoleate and oleate. By causing formation of greater amounts of these monounsaturated compounds, this invention also favors the production of other fatty acid moieties of higher degrees of desaturation for which the monounsaturates serve as precursors. These unsaturated moieties are identical to naturally occurring unsaturated fatty acid moieties and can then be incorporated into triacylglycerol storage lipids in the plant tissues via existing biochemical pathways. In this way the percentage composition of the unsaturated fatty acid moieties and the corresponding triacylglycerols (as well as mono- and diacylglycerols, phospholipids, and other fatty acid-derived lipids) is increased. Thus, this invention also provides plant cells and whole plants having increased percentage compositions of unsaturated fatty acid moieties and corresponding fatty acid-derived lipids, in which the plant cells contain a chimeric gene construct according to this invention. Also provided are methods for increasing the percentage compositions of unsaturated fatty acid moieties and corresponding fatty acid-derived lipids in plant cells and whole plants, comprising the step of inserting into such plant cells or the cells of such whole plants a chimeric gene construct according to this invention.

[0018] Considering the tobacco plant as a model system, there are two major saturated fatty acids found in tobacco leaves, palmitic (16:0) and stearic (18:0) acids. Using these molecules as substrates, double bonds are formed one at a time by consecutive desaturation reactions. These reactions involve desaturases, which catalyze hydrogen removal, and electron transport components. In plants, the first desaturation step is catalyzed by a soluble enzyme localized in chloroplasts. In higher plants, 16:1 and 18:1 are formed from palmitic and stearic acids esterified to acyl carrier protein (ACP) or certain glycerol lipids. Subsequent desaturation reactions can occur in both plastids and the endoplasmic reticulum, with different genes apparently encoding the enzymes found in the different subcellular locations. It has been estimated that there are at least eight genes controlling the activity of specific desaturases in the plant system.

[0019] In vitro studies of safflower desaturase show a requirement for ferrodoxin added to *E. coli* extracts. Similar results were found for avocado desaturase.

[0020] In a preferred embodiment of this invention, the stearyl coenzyme A desaturase from rat is used. This is a $\Delta 9$ desaturase that in the normal rat requires electrons from reduced cytochrome b_5 in the cytoplasm. However, in the normal plant these reactions normally occur via a $\Delta 9$ desaturase in the chloroplast. Intermembrane forms of fatty acids in the cytoplasm can exist as CoA derivatives. It has now been discovered that a desaturase from an organism in another kingdom can function effectively with essential biopathway components supplied by a plant despite the wide divergence between the gene and protein sequences and structures in the two kingdoms. Thus, this invention involves creation of a biochemical pathway in the plant that normally uses the CoA forms of fatty acids and functions in the cytoplasm rather than the chloroplast. The results obtained with this invention indicate that cytochrome b_5 and cytochrome b_5 reductase present in plants can substitute for these corresponding components normally found in the functional rat desaturase complex.

[0021] It has also been observed that the synthesis of unsaturated fatty acids and the lipids derived therefrom is a common plant response to cold environmental conditions. Bacteria with altered membrane fatty acid compositions have been shown to survive at lower temperatures than wild-type strains. While not intending to be limited by theory, it is believed that synthesis of these fatty acids and lipids, which remain liquid at lower temperatures than their saturated counterparts and therefore help to maintain membrane fluidity at low temperatures, helps the plant to tolerate those lower temperatures. Thus, the present invention also offers the opportunity to enhance the cold tolerance of plants by augmenting their natural ability to synthesize unsaturated fatty acids and lipids derived therefrom.

[0022] Conversely, it will be understood that plants also tend to synthesize fully saturated fatty acids and lipids derived therefrom under warmer environmental conditions. On the other hand, the desaturase genes used in this invention are typically derived from animals or yeasts, and therefore function quite effectively at the warmer temperatures normally provided by those natural host organisms. Thus, the present invention also provides the ability to offset or compensate for the effects of warmer growing temperatures on lipid composition in oilseed plants by providing a desaturase gene which is more active at higher temperatures and thus produces greater quantities of fatty acid desaturation products with increasing temperature.

Industrial Applicability

[0023] While any plant species can be modified using the expression cassette and methods of this invention, including without limitation species from the genera *Fragaria*, *Lotus*, *Medicago*, *Onobrychis*, *Trifolium*, *Trigonella*, *Vigna*, *Citrus*, *Linum*, *Geranium*, *Manicot*, *Daucus*, *Arabidopsis*, *Brassica*, *Raphanus*, *Sinapis*, *Atropa*, *Capsicum*, *Datura*, *Hyoscyamus*, *Lycopersicon*, *Nicotiana*, *Solanum*, *Petunia*, *Digitalis*, *Majorana*, *Cichorium*, *Helianthus*, *Lactuca*, *Bromus*, *Asparagus*, *Antirrhinum*, *Hemerocallis*, *Nemesia*, *Pelargonium*, *Panicum*, *Pennisetum*, *Ranunculus*, *Senecio*, *Salpiglossis*, *Cucumis*, *Browallia*, *Glycine*, *Lolium*, *Triticum*, and *Datura*, the fatty acid modifications provided by this invention are expected to be used to best advantage in oil-bearing crops such as soybean, sunflower, rapeseed, safflower, peanut, corn, flax, Chinese tallow tree, jojoba, and palm.

[0024] The fatty acid-Coenzyme A desaturase gene can be cloned from any suitable animal or yeast host, since it has been determined that the sequence of this gene is highly conserved across mammalian species and there are even regions of homology between mammalian and yeast genes. Genes encoding the desaturase catalyzing the 18:0 to 18:1 step have been cloned from rat, mouse, and yeast. The Human Genome Project will ultimately produce the coding sequence for the human fatty acid-CoA desaturase, which can be used if desired. On the other hand, no detectable identity has been found between the amino acid sequence of the rat stearyl CoA desaturase (used in the transformation work described herein) and castor (*Ricinus communis*) stearyl-ACP desaturase, so the needed Coenzyme A-dependent gene cannot be obtained from plants.

[0025] By "fatty acid-derived lipids" herein is meant any naturally occurring plant lipid which has as a precursor a saturated or unsaturated C12 or higher fatty acid synthesized in the plant. These include, without limitation, mono-, di- and triacylglycerols, phospholipids such as phosphatidylcholine and Phosphatidylethanolamine, glycolipids and other lipids of leafy tissues, and others. By converting the naturally occurring fatty acid "feedstocks" for the synthesis of these compounds from saturated to unsaturated, this invention causes the natural biochemical pathways present in the plant to

favor the production of the corresponding unsaturated fatty acid-derived lipids.

[0026] The following examples illustrate the practice of the present invention without intending to be limitative thereof.

Example 1

Construction of the plant expression plasmid.

[0027] The cDNA for stearyl CoA desaturase from rat consists of a 1.1 kb coding sequence followed by a 3.5 kb untranslated sequence. The gene used in the examples herein codes for amino acids 3 to 356. The plasmid pDs3-358, constructed as described in *J. Biol. Chem.*, 263:2532-2536, was provided by P. Strittmatter. This was digested with BamHI and SstI to release a 1.2 kb fragment that contained the rat desaturase gene. This was ligated to Bluescript SK+ (Stratagene) digested with BamHI and SstI. This plasmid was then digested with HindIII and SstI to release a 1.2 kb fragment. The plant expression vector pKYLX71:35S is a derivative of pKYLX71 in which the CaMV 35S promoter was replaced with a 35S promoter containing a duplication of bases -416 to -90 relative to the transcription initiation site. This plasmid was digested with HindIII and SstI, treated with calf intestinal alkaline phosphatase, and ligated to the 1.2 kb fragment described above to create p71²RDS. In addition to the structural gene for rat desaturase, this plasmid contains the neomycin phosphotransferase gene (conferring kanamycin resistance), a broad host range RK2 replication origin, T-DNA borders, and a polyadenylation signal. A map of this plasmid is shown in FIG. 1. The control plasmid lacked the rat desaturase structural gene but was otherwise identical. Competent *E. coli* TB1 were transformed with the plasmids. Single colonies were recovered from LB plates supplemented with 50 mg/L kanamycin and 12.5 mg/L tetracycline. Colonies were transferred to liquid cultures and DNA was prepared by alkali lysis. Plasmids with the expected size were used for further experiments.

Example 2

Plant Transformation.

[0028] Plants of *Nicotiana tabacum* cv. 'Xanthi' were transformed using cocultivation with *Agrobacterium tumefaciens* as generally practiced (see, e.g. the 1985 article by Horsch et al., *Science* 227:1229-12231) except leaf strips were used instead of disks. Selection for resistance to kanamycin was at done at a concentration of 100 mg/L. Callus induction, shooting, and rooting all occurred in the presence of kanamycin. Mefoxin was included in media at 300 mg/L to inhibit *Agrobacterium tumefaciens* division.

[0029] The plasmids pKYLX71:35S and p71²RDS were mobilized from *E. coli* to *A. tumefaciens* strain LBA4404 using triparental crosses using pRK2013 as the helper plasmid.

Example 3

Plant DNA Isolation and Sequence Amplification.

[0030] Total plant DNA was isolated from green leaves using acidified guanidinium thiocyanate extraction. After total plant nucleic acids were mixed in 4 M LiCl and centrifuged, the supernatant (that contained the DNA) was removed to a new tube and precipitated with two volumes of ethanol. DNA was then centrifuged, washed with 70% ethanol, dried under vacuum, and resuspended in 1 mM Tris, 0.1 mM EDTA pH 7.5.

[0031] PCRs were performed in a total volume of 20 μ L and contained 200 ng total leaf DNA, 11 ng primers (5' ACGTGGATCCACCATGCCGCGCCACATGCTC 3') and (5' GCTACTCTTGTGGCTCCC 3'), 1 mM dATP, dCTP, dGTP, and dTTP, 50 mM KCl, 10 mM Tris pH 8.3, 1.5 mM MgCl₂, 0.01% gelatin, and 1 unit of Taq DNA polymerase (AmpliTaqTM DNA polymerase, Perkin-Elmer Cetus). A negative control consisted of all the same components with the exception of the plant DNA. Another negative control included DNA from kanamycin resistant plants transformed with the vector used for plant transformation (pKYLX71:35S) but without the desaturase sequence. Reaction mixtures were covered with mineral oil and placed in a thermal cycler (Lab-Line). The temperature was cycled to 95°C for 2 sec (denaturation), then to 64°C and 59°C each for 2 sec (annealing), and then to 72°C for 180 sec (extension) for a total of 30 cycles. PCR products were fractionated on a 0.8% agarose gel in TAE running buffer. Size markers were 1 kb DNA ladders (BRL).

Example 4

Lipid Fractionation and Fatty Acid Analysis.

[0032] Leaf tissue (approximately 0.5 g) was placed in 3 mL chloroform:methanol (2:1) and ground in a Tissumizer

(Tekmar). Solvents were then concentrated in a Speedvac and fractionated on a silica gel 60 plate for PC isolation. Spots were scraped and methyl esters were prepared in sulfuric acid/methanol as described below. Leaf samples used for dry weight calculations were lyophilized.

[0033] Methyl esters of total fatty acids were prepared in glass tubes from 100-200 mg of callus tissue that was ground in 2 mL 1% sulfuric acid in methanol with a tissumizer as described above or with glass rods. Leaves prepared for total fatty acid analysis were not ground. Transesterification proceeded at 80°C until approximately 0.2 mL of the methanol remained. Heptadecanoic acid (17:0; 1 mg/mL in hexane) was added as an internal standard directly to plant tissue at 0.1 mg per gram tissue fresh weight. Hexane was allowed to evaporate for about 10 min. prior to tissue extraction. Quantitation of total plant fatty acids was determined by a comparison of peak areas on chromatograms with heptadecanoic acid areas. For quantitation of fatty acids from lipids fractionated on TLC plates, 25 mg nonadecanoic acid (19:0) was added to each spot prior to scraping. Fatty acid methyl esters were analyzed by gas chromatography as described by Dahmer et al., *J. Amer. Oil Chem. Soc.* 66:543-548 (1989) except that a Hewlett Packard FFAP column was used.

[0034] Standards for PC, FFA, TG, 16:0, etc. were from Sigma. Leaves from the FadC mutant of *Arabidopsis thaliana* can accumulate 16:1 [Science 252:80-87 (1991)]. FadD mutant leaves can accumulate 16:2 with a corresponding reduction in 16:3. Extracts from these and wild type leaves were used to determine the retention times for 16:1, 16:2 and 16:3.

RESULTS

[0035] Following *Agrobacterium tumefaciens*-mediated transformation, kanamycin-resistant tobacco calli were screened for altered fatty acid composition. Several calli had higher ratios of 16:1/16:0 and/or 18:1/18:0 than controls (FIG. 2). The ratio of 18:1/18:0 could not be predicted from the 16:1/16:0 ratio. In nine of the ten desaturase transformants examined, the 18:1/18:0 ratio was higher than the 16:1/16:0 ratio, as was also seen in control calli. These data suggested that regenerated plants might also have altered fatty acid levels.

[0036] Data indicating the presence of the introduced desaturase gene are presented in FIG. 3. PCR amplification of total plant DNA confirmed that the expected 1.1 kb product is not seen in 2 control plants transformed with a plasmid that lacks the rat desaturase gene. FIGS. 4 through 7 graphically depict data obtained from analysis of leaves from these same plants.

[0037] As with calli, leaves harboring the rat desaturase gene were hoped to have an increase in the 16:1/16:0 and/or the 18:1/18:0 ratios when compared to controls. FIG. 4 shows a clear increase in the 16:1/16:0 ratio for two plants (RT and RU) transformed with the rat desaturase gene. Plant RT has a higher 18:1/18:0 ratio than controls, while RU does not. This is in contrast to data from calli (FIG. 2), where 18:1/18:0 ratios were consistently higher in calli carrying the rat desaturase gene than in controls. However, the CaMV 35S promoter does not work as well in some differentiated tissues as it does in undifferentiated callus cells, and that may explain the difference.

[0038] Since it was unknown what effect an introduced desaturase might have on fatty acid composition and levels, quantities of 16:0, 16:1, 16:2, 16:3, 18:0, 18:1, 18:2, and 18:3 were also determined. To visualize how these moieties changed, the amounts of 16:0, 16:1, 16:2, 16:3, 18:0, 18:1, 18:2, and 18:3 in tobacco leaves were combined and the amount of each fatty acid was expressed as a percentage of this total (FIG. 5). A large increase in 16:1 as well as a reduction in 16:0 and 18:0 can be seen in plants RT and RU. Levels of 18:2 also increased relative to controls. The desaturase transformants RT and RU also have a reduced percentage of 18:3 when compared to controls.

[0039] In order to obtain an overall view of lipid accumulation, 16 and 18 carbon fatty acids (16:0, 16:1, 16:2, 16:3, 18:0, 18:1, 18:2, and 18:3) were combined and expressed as mg FA per gram fresh weight or dry weight (data not shown). The similarity between total 16 and 18 carbon fatty acid levels in control and desaturase-transformed plants suggests a compensation between pathways involved in leaf lipid metabolism. Such a compensation has been implicated in various desaturase mutants of *Arabidopsis* [Science 252:80-87 (1991)].

[0040] To further indicate that elevated levels of 16:1 found in desaturase-transformed tobacco resulted from the product of the introduced gene, leaf lipids were fractionated into classes. Since 16:1 does not normally appear in plant phosphatidylcholine (PC), we examined this fraction in more detail, with results as illustrated in FIG. 6. It can be seen that 16:1 was only detected in PC from plants transformed with the rat desaturase gene. This qualitative difference between controls and desaturase transformants provides clear evidence that the introduced desaturase is functioning in leaf tissue. The presence of 16:1 in phosphatidylcholine from leaves transformed with the rat desaturase gene (FIG. 6) is a necessary requirement for changing seed lipid composition, which is primarily triglyceride. Phosphatidylcholine is the immediate precursor of triglyceride in developing seeds.

[0041] The ability to manipulate fatty acid composition in plants with a desaturase from rat demonstrates that an approach other than mutant selection can be used to alter plant lipid levels.

Example 5

[0042] Seed from transgenic plants has also been examined and the dramatic increase in 16:1 fatty acid moieties in total leaf lipids is echoed in seed lipid, although at a lower level, as seen in FIG. 7. The desaturated/saturated fatty acid ratio has also been increased in representative desaturase transformants relative to control transgenic plant leaf and seed tissues (FIG. 7). This is further illustrated by observation of the saturated-to-unsaturated ratio of 16-carbon fatty acid moieties. As seen in FIG. 8, selected transgenic plants G, M, U, T, J, and W had large reductions in 16:0/(16:1 + 16:2 + 16:3) compared to control plant E.

Example 6

[0043] The plasmid pDs3-358, constructed as described in J. Biol. Chem., 263:2532-2536, was provided by P. Strittmatter. This was digested with BamHI and SstI to release a 1.2 kb fragment that contained the rat desaturase gene. This was ligated to a previously made ACT vector, pALLNAPG1, to generate pALLNAPSCD; this process juxtaposed the stearyl CoA desaturase coding portion directly behind the seed-specific promoter napin in a binary vector, ready for plant transformation.

[0044] A promoter-gene cassette carrying the napin promoter and stearyl CoA desaturase gene was excised as a HindIII-SstI fragment and used to replace the HindIII-SstI fragment of a binary vector, pALLTKrep, to generate pALLTKNAPSCD, which was structurally and functionally equivalent to pALLNAPSCD except it provided cleaner selection for transgenic plants on kanamycin antibiotic. Transfer of the DNA into *Agrobacterium* was accomplished by transformation directly into the organism, as opposed to triparental mating as described above. The gene was inserted into canola cells by cell wounding and cocultivation, and whole canola transformants were regenerated from the transformed callus cultures. Seeds were obtained from the resulting regenerated transformed plants.

SEQUENCE LISTING

INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 bases
- (B) TYPE: nucelotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(x) PUBLICATION INFORMATION:

- (A) AUTHORS: Stritmatter et al.
- (C) JOURNAL: J. Biol. Chem.

(D) VOLUME: 263

(F) PAGES: 2532-2536

(G) DATE: 1988

(xi) SEQUENCE DESCRIPTION: SEQ. ID. NO. 1:

ACGTGGATCC ACCATGCCGG CCCACATGCT C

31

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 bases
- (B) TYPE: nucelotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: Yes

(x) PUBLICATION INFORMATION:

(A) AUTHORS: Stritmatter et al.

(C) JOURNAL: J. Biol. Chem.

(D) VOLUME: 263

(F) PAGES: 2532-2536

(G) DATE: 1988

(xi) SEQUENCE DESCRIPTION: SEQ. ID. NO. 2:

GCTACTCTTG TGGCTCCC

18

Claims

1. A chimeric expression cassette comprising a cDNA clone which encodes a mammalian fatty acid-Coenzyme A desaturase, operably linked to regulatory sequences which cause the expression of the cDNA clone in plant cells.
2. An expression cassette according to claim 1 wherein the fatty acid-Coenzyme A desaturase gene is a stearyl-Coenzyme A desaturase gene.
3. An expression cassette according to claim 1 or claim 2 wherein the fatty acid-Coenzyme A desaturase gene is a

stearyl-Coenzyme A desaturase gene from the rat.

4. An expression cassette according to any one of the preceding claims, which is capable of being replicated in a bacterial cell.
5. Bacterial cells containing as a foreign plasmid at least one copy of an expression cassette according to claim 4.
6. Transformed plant cells containing at least one copy of the expression cassette according to any one of claims 1 to 3.
7. Transformed cells according to claim 6, further characterized in being cells of a monocotyledonous species.
8. Transformed cells according to claim 7, further characterized in being maize, sorghum, wheat, palm or rice cells.
9. Transformed cells according to claim 6, further characterized in being cells of a dicotyledonous species.
10. Transformed cells according to claim 9, further characterized in being soybean, rapeseed, jojoba, Chinese tallow tree, tobacco, safflower, peanut, or sunflower cells.
11. A cell culture or tissue culture comprising cells according to any one of claims 5 to 10.
12. A method of increasing the percentage compositions of unsaturated fatty acid moieties and corresponding fatty acid-derived lipids in plant cells and whole plants, comprising the step of inserting into such plant cells or the cells of such whole plants a chimeric expression cassette according to any one of claims 1 to 3.
13. A method according to claim 12 which comprises the further step of sexually or clonally reproducing the whole plants in such manner that at least one copy of the sequence provided by the expression cassette is present in the cells of progeny of the reproduction.
14. A method according to claim 12 in which the expression cassette is introduced into the cells by electroporation, microparticle bombardment or microinjection.
15. A method for increasing the percentage compositions of unsaturated fatty acid moieties and corresponding fatty acid-derived lipids in Agrobacterium-susceptible dicotyledonous plants in which the expression cassette is introduced into the cells by infecting the cells with Agrobacteria, a plasmid of which has been modified to include the expression cassette according to any one of claims 1 to 3.

Patentansprüche

1. Chimäre Expressionskassette, umfassend einen cDNA-Klon, welcher für eine Säuger-Fettsäure-Coenzym A-Desaturase kodiert, in funktionsfähiger Verknüpfung mit regulatorischen Sequenzen, welche die Expression des cDNA-Klons in Pflanzenzellen veranlassen.
2. Expressionskassette nach Anspruch 1, worin das Fettsäure-Coenzym A-Desaturase-Gen ein Stearyl-Coenzym A-Desaturase-Gen ist.
3. Expressionskassette nach Anspruch 1 oder Anspruch 2, worin das Fettsäure-Coenzym A-Desaturase-Gen ein Stearyl-Coenzym A-Desaturase-Gen aus der Ratte ist.
4. Expressionskassette nach irgendeinem der vorherigen Ansprüche, welche imstande ist, in einer Bakterienzelle repliziert zu werden.
5. Bakterienzellen, welche mindestens eine Kopie einer Expressionskassette nach Anspruch 4 als fremdes Plasmid enthalten.
6. Transformierte Pflanzenzellen, welche mindestens eine Kopie der Expressionskassette nach irgendeinem der Ansprüche 1 bis 3 enthalten.

7. Transformierte Zellen nach Anspruch 6, ferner dadurch gekennzeichnet, daß es sich um Zellen einer einkeimblättrigen Spezies handelt.
- 5 8. Transformierte Zellen nach Anspruch 7, ferner dadurch gekennzeichnet, daß es sich um Zellen von Mais, Sorghum, Weizen, Palme oder Reis handelt.
9. Transformierte Zellen nach Anspruch 6, ferner dadurch gekennzeichnet, daß es sich um Zellen einer zweikeimblättrigen Spezies handelt.
- 10 10. Transformierte Zellen nach Anspruch 9, ferner dadurch gekennzeichnet, daß es sich um Zellen von Sojabohne, Rapssamen, Jojoba, Chinesischem Lichtnußbaum, Tabak, Saflor, Erdnuß oder Sonnenblume handelt.
11. Zellkultur oder Gewebekultur, umfassend Zellen nach irgendeinem der Ansprüche 5 bis 10.
- 15 12. Verfahren zur Erhöhung der prozentualen Zusammensetzungsanteile ungesättigter Fettsäuregruppierungen und entsprechender Fettsäure-abgeleiteter Lipide in Pflanzenzellen und ganzen Pflanzen, umfassend den Schritt der Einführung einer chimären Expressionskassette nach irgendeinem der Ansprüche 1 bis 3 in solche Pflanzenzellen oder in die Zellen solcher ganzen Pflanzen.
- 20 13. Verfahren nach Anspruch 12, umfassend den weiteren Schritt der sexuellen oder klonalen Reproduktion der ganzen Pflanzen auf solche Weise, daß mindestens eine Kopie der von der Expressionskassette bereitgestellten Sequenz in den Zellen der Nachkommenschaft der Reproduktion vorhanden ist.
- 25 14. Verfahren nach Anspruch 12, worin die Expressionskassette durch Elektroporation, Mikroteilchenbeschuß oder Mikroinjektion in die Zellen eingeführt wird.
- 30 15. Verfahren zur Erhöhung der prozentualen Zusammensetzungsanteile ungesättigter Fettsäuregruppierungen und entsprechender Fettsäure-abgeleiteter Lipide in für ein Agrobakterium anfälligen zweikeimblättrigen Pflanzen, worin die Expressionskassette in die Zellen eingeführt wird durch Infektion der Zellen mit Agrobakterien, in denen ein Plasmid modifiziert wurde, um die Expressionskassette nach irgendeinem der Ansprüche 1 bis 3 einzuschließen.

Revendications

- 35 1. Cassette d'expression chimère comprenant un clone d'ADNc codant pour une acide gras - coenzyme A désaturase de mammifère, liée de façon opérationnelle aux séquences régulatrices entraînant l'expression du clone d'ADNc dans les cellules d'une plante.
- 40 2. Cassette d'expression selon la revendication 1, dans laquelle le gène d'acide gras - coenzyme A désaturase est un gène de stéaryl - coenzyme A désaturase.
3. Cassette d'expression selon la revendication 1 ou la revendication 2, dans laquelle le gène d'acide gras - coenzyme A désaturase est le gène de la stéaryl - coenzyme A désaturase du rat.
- 45 4. Cassette d'expression selon une quelconque des revendications précédentes, qui est capable de se répliquer dans une cellule bactérienne.
5. Cellules bactériennes contenant en tant que plasmide étranger au moins une copie d'une cassette d'expression selon la revendication 4.
- 50 6. Cellules végétales transformées contenant au moins une copie d'une cassette d'expression selon une quelconque des revendications 1 à 3.
7. Cellules transformées selon la revendication 6, caractérisées en outre en ce qu'il s'agit de cellules d'une espèce de monocotylédone.
- 55 8. Cellules transformées selon la revendication 7, caractérisées en outre en ce qu'il s'agit de cellules de maïs, de sorgho, de blé, de palmier ou de riz.

9. Cellules transformées selon la revendication 6, caractérisées en outre en ce qu'il s'agit de cellules d'une espèce de dicotylédone.

10. Cellules transformées selon la revendication 9, caractérisées en outre en ce qu'il s'agit de cellules de soja, de colza, de jojoba, d'arbre à suif, de tabac, de carthame, d'arachide ou de tournesol.

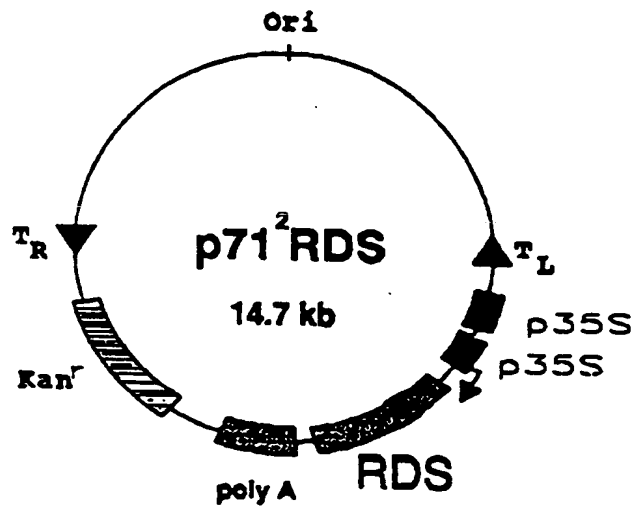
11. Culture de cellules ou culture de tissus comprenant des cellules selon une quelconque des revendications 5 à 10.

12. Méthode pour augmenter les compositions en pourcentage des fragments d'acides gras insaturés et des lipides dérivés d'acides gras correspondants dans les cellules de plantes et les plantes entières, comprenant l'étape d'insertion, dans de telles cellules de plantes ou dans les cellules de telles plantes entières, d'une cassette d'expression chimère selon une quelconque des revendications 1 à 3.

13. Méthode selon la revendication 12, comprenant l'étape supplémentaire de reproduction sexuée ou par clonage des plantes entières de telle façon qu'au moins une copie de la séquence fournie par la cassette d'expression soit présente dans les cellules de la descendance de la reproduction.

14. Méthode selon la revendication 12, dans laquelle la cassette d'expression est introduite dans les cellules par électroporation, bombardement par microparticules ou microinjection.

15. Méthode pour augmenter les compositions en pourcentage des fragments-acides gras insaturés et des lipides dérivés d'acides gras correspondants dans des plantes dicotylédones sensibles à *Agrobacterium* dans les cellules desquelles une cassette d'expression est introduite par infection des cellules par des agrobactéries dont un plasmide a été modifié pour inclure une cassette d'expression selon une quelconque des revendications 1 à 3.



Map of plasmid for expression of
Rat Desaturase (RDS) in Tobacco

FIG. 1

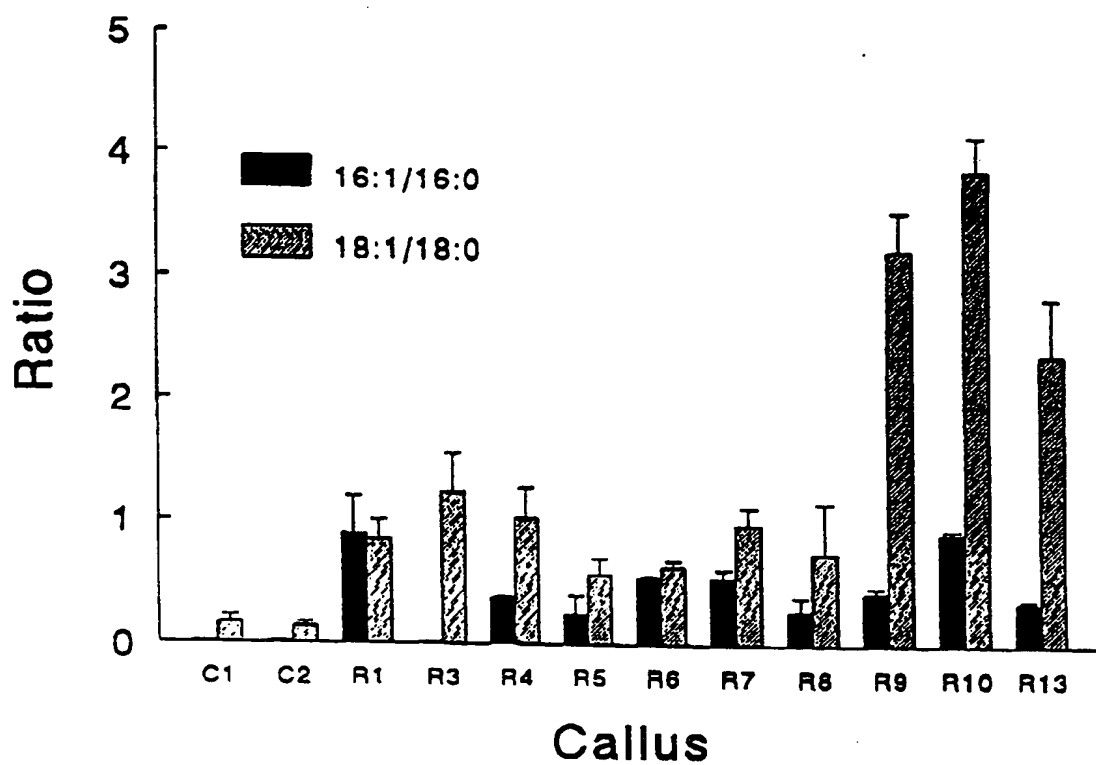


FIG. 2

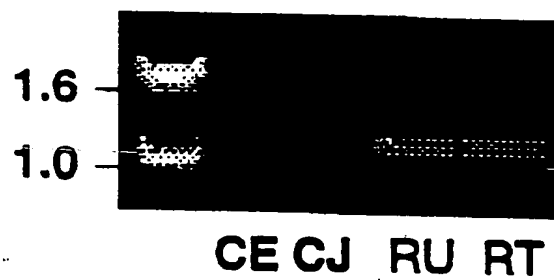


FIG. 3

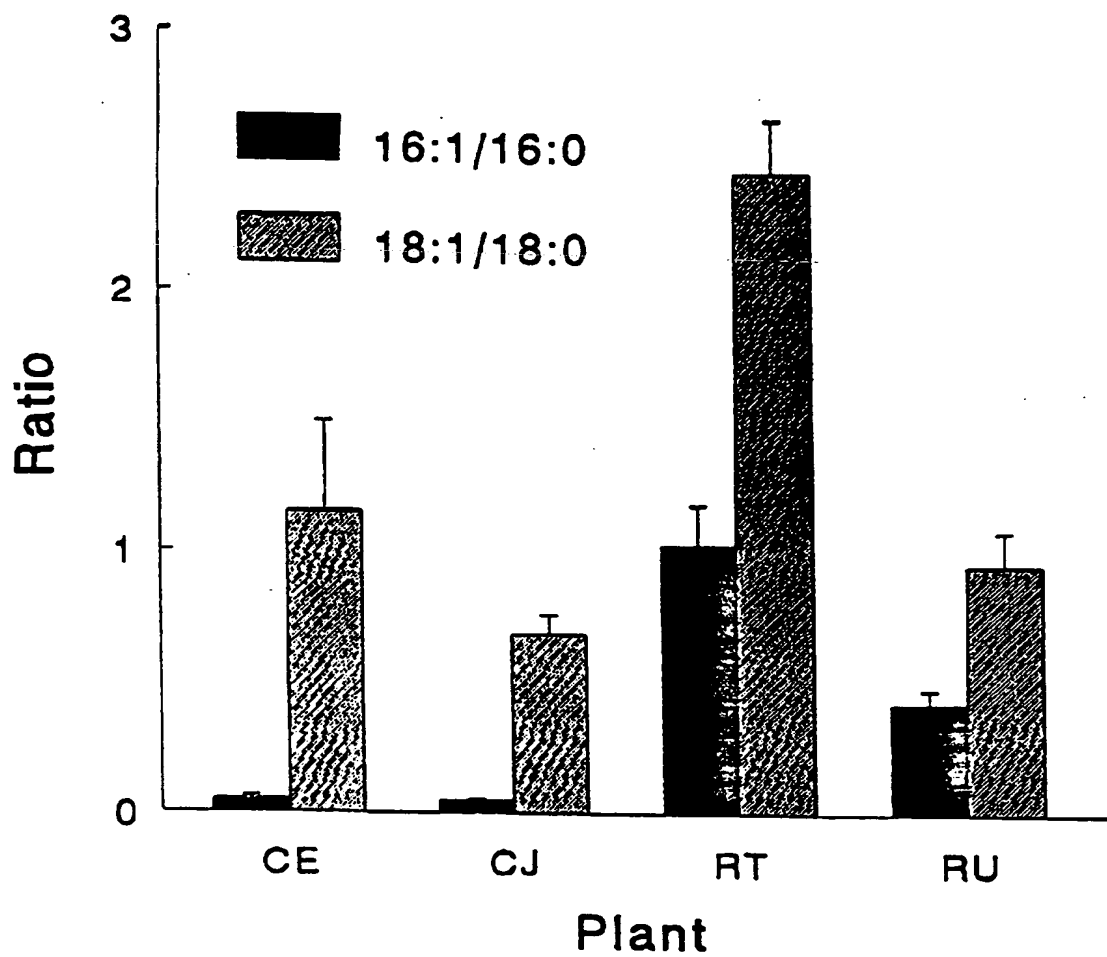


FIG. 4

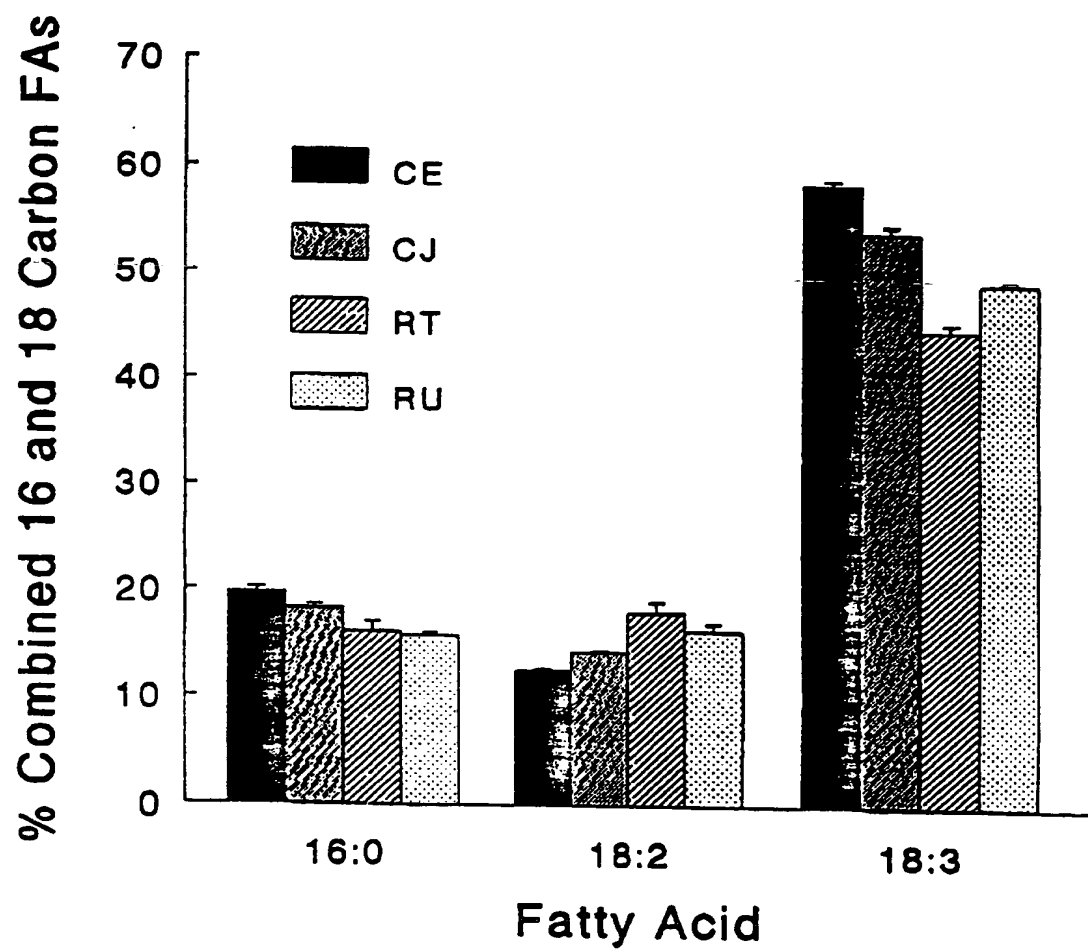


FIG. 5

